Alginate Production by *Pseudomonas putida* Creates a Hydrated Microenvironment and Contributes to Biofilm Architecture and Stress Tolerance under Water-Limiting Conditions[▽]

Woo-Suk Chang,¹† Martijn van de Mortel,¹ Lindsey Nielsen,¹ Gabriela Nino de Guzman,²
Xiaohong Li,² and Larry J. Halverson^{1,2*}

Graduate Program in Microbiology¹ and Department of Plant Pathology,² Iowa State University, Ames, Iowa 50011

Received 9 May 2007/Accepted 20 June 2007

Biofilms exist in a variety of habitats that are routinely or periodically not saturated with water, and residents must integrate cues on water abundance (matric stress) or osmolarity (solute stress) into lifestyle strategies. Here we examine this hypothesis by assessing the extent to which alginate production by *Pseudomonas putida* strain mt-2 and by other fluorescent pseudomonads occurs in response to water limitations and how the presence of alginate in turn influences biofilm development and stress tolerance. Total exopolysaccharide (EPS) and alginate production increased with increasing matric, but not solute, stress severity, and alginate was a significant component, but not the major component, of EPS. Alginate influenced biofilm architecture, resulting in biofilms that were taller, covered less surface area, and had a thicker EPS layer at the air interface than those formed by an mt-2 *algD* mutant under water-limiting conditions, properties that could contribute to less evaporative water loss. We examined this possibility and show that alginate reduces the extent of water loss from biofilm residents by using a biosensor to quantify the water potential of individual cells and by measuring the extent of dehydration-mediated changes in fatty acid composition following a matric or solute stress shock. Alginate deficiency decreased survival of desiccation not only by *P. putida* but also by *Pseudomonas aeruginosa* PAO1 and *Pseudomonas syringae* pv. *syringae* B728a. Our findings suggest that in response to water-limiting conditions, pseudomonads produce alginate, which influences biofilm development and EPS physiochemical properties. Collectively these responses may facilitate the maintenance of a hydrated microenvironment, protecting residents from desiccation stress and increasing survival.

Bacteria can colonize a variety of habitats as biofilms. In many terrestrial habitats such as soil or plant surfaces, in particular, these biofilms may not be saturated, or at least may not be consistently saturated, with water. The thickness of the water film surrounding these unsaturated biofilms will vary depending on environmental conditions. When the biofilms are fully saturated, the total water potential (ψ) (free energy of water) is composed almost exclusively of the solute potential. As water is lost from the habitat, i.e., as the habitat becomes drier, the matric potential becomes the predominant factor contributing to the total water potential (32). These stresses differ in that, with a solute stress, bacteria are bathed in water of diminished activity, but with a matric stress, bacteria become desiccated by physical removal of water from their environment, and the availability of the remaining water is reduced through its interaction with the matrix. Relatively little is known about how water availability influences biofilm development and how biofilm properties in turn influence bacterial survival under conditions typically encountered in terrestrial ecosystems.

Unsaturated *Pseudomonas* biofilm formation is a dynamic process where reduced water availability affects biofilm devel-

opment and ultrastructural properties (1, 8, 43). As in fully hydrated, flowthrough systems, biofilms in unsaturated habitats are encapsulated by an exopolysaccharide (EPS) layer (8, 33). Since many EPSs are hygroscopic, their presence presumably creates a more hydrated microenvironment in the immediate vicinity of the cells, thereby contributing to desiccation tolerance (31, 36, 39). Roberson and Firestone (36) observed more EPS production by a soil *Pseudomonas* sp. in desiccated than in undesiccated sand cultures, suggesting that resources were allocated to EPS production in response to desiccation. Although the increased EPS production presumably protects cells from desiccation stress, surprisingly, there is relatively little evidence to support the notion that *Pseudomonas* EPS, or a particular EPS constituent, ameliorates the stresses bacterial cells actually experience under desiccating conditions.

The fluorescent pseudomonads have the potential to produce many different types of EPS constituents, including, for example, levan, marginalan, cellulose, and alginate, in addition to several uncharacterized polymers (12, 13, 19, 20, 25, 33). Despite the nearly universal ability of *Pseudomonas* species to produce alginate, a class of polymers comprising the uronic acids D-mannuronic and/or its epimer L-guluronic acid assembled into β -1,4-linked blocks that can be O acetylated (37, 44), the exact benefits of alginate production have been elusive. Potential functions have been indicated by examining alginate biosynthesis structural (22, 53) or regulatory (39, 49) mutants or by identifying environmental factors influencing the expression of alginate genes (9, 21, 34, 46). *Pseudomonas aeruginosa* and *Pseudomonas syringae* share conserved signals, including

* Corresponding author. Mailing address: Department of Plant Pathology, Iowa State University, Ames, IA 50011-1010. Phone: (515) 294-0495. Fax: (515) 294-6019. E-mail: larryh@iastate.edu.

† Present address: Division of Plant Sciences and Microbiology, 201 Life Sciences Center, University of Missouri, Columbia, MO 65211.

[▽] Published ahead of print on 29 June 2007.

TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Genotype, phenotype, or sequence ^a	Reference or source
Strains		
<i>P. putida</i>		
mt-2		46
mt-2 <i>algD</i>	<i>algD</i> ::pKAD; Gm ^r ; alginate-deficient mutant	This study
<i>P. aeruginosa</i>		
PAO1	Wild type	M. Parsek
PAO1 <i>algD</i>	<i>algD</i> deletion mutant	49
<i>P. syringae</i> pv. <i>syringae</i>		
B728a	Wild type	G. Beattie
B728a <i>algD</i>	<i>algD</i> deletion mutant	G. Beattie
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80 <i>dlacZ</i> Δ <i>M15</i> Δ (<i>lacZYA-argF</i>) <i>U169 deoR recA1 endA1 hsdR17 phoA supE44</i> λ ⁻ <i>thi-1 gyrA96 relA1</i>	Gibco-BRL
S17-1	RP4 transfer function on chromosome	40
TOP10	F ⁻ <i>mcrA</i> (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ <i>M15 lacX74 deoR nupG recA1 araD139</i> (<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (Sm ^r) <i>endA1</i>	Invitrogen
Plasmids		
pRK2073	RP4 transfer function on plasmid; Sm ^r	14
pTOPO PCR 2.1	Cloning vector	Invitrogen
pTOPO-Pal <i>gD</i>	pTOPO PCR2.1 with <i>algD</i> operon promoter	This study
p <i>NptII-gfp</i>	pPROBE-KT carrying an <i>nptII-gfp</i> fusion	8
pPROBE-NT	Promoter probe vector with promoterless <i>gfp</i> , pBBR1 <i>ori</i>	28
p <i>PalgD-gfp</i>	<i>algD</i> operon promoter cloned into pPROBE-NT	This study
pKnockout-G	Suicide vector; Gm ^r	47
pKAD	Truncated <i>algD</i> (622 bp) in pKnockout-G	This study
pP <i>ProIce</i>	Plasmid containing an inducible <i>proU-inaZ</i> fusion	50
pP <i>NptIce</i>	Plasmid containing a constitutive <i>nptII-inaZ</i> fusion	50
Primers		
algDF	5'-TTGGTTTGGGTTATGTGGG-3'	This study
algDR	5'-CAGGTGTACTTGATCATTTCGG-3'	This study
pKOF	5'-CCCAGTCACGACGTTGTAAAACG-3'	This study
pKOR	5'-AGCGGATAACAATTTACACAGG-3'	This study
AlgDpr <i>m</i> -F	5'-TGAAGCCGCGAAGAGGTCAG-3'	This study
AlgDpr <i>m</i> -R	5'-CGCATCGCTATCACCTCATG-3'	This study

^a Rif^r, rifampin resistance; Gm^r, gentamicin resistance; Sm^r, streptomycin resistance.

elevated osmolarity and elevated levels of reactive oxygen species, for activating transcription from alginate promoters, yet neither requires alginate for biofilm formation in flowthrough systems (25, 49). This was particularly surprising for *P. aeruginosa*, since it was widely held that alginate was necessary for biofilm formation, the dominant lifestyle in the cystic fibrosis (CF) lung (41). The chaotropic permeating solute ethanol has been shown to increase alginate production in some fluorescent pseudomonads (42) and to induce expression of the *P. aeruginosa algD* promoter; in the latter case, ethanol did not increase alginate production but did increase the frequency of mucoid variants (9). Although deletion of the global regulator σ^U decreased the EPS production and desiccation tolerance of *Pseudomonas fluorescens* (39), the extent to which the lack of EPS production or of a particular EPS constituent was responsible for the decreased tolerance was not demonstrated. We recently showed that the alginate biosynthesis structural genes were induced by water limitation, but not by high osmolarity, in the soil bacterium *Pseudomonas putida* (46).

In this report, we provide direct evidence that alginate func-

tions to maintain cellular hydration, a function that has long been assumed and predicted but not demonstrated. Moreover, alginate does this even when it is not a major component of the EPS matrix. We also provide evidence that alginate is integral to biofilm architecture under water-limiting or matric stress conditions, but interestingly, it is not integral to biofilm architecture under solute stress or water-replete conditions. Collectively, our findings indicate that alginate facilitates the maintenance of a hydrated biofilm microenvironment and biofilm architecture that protect cells of *P. putida* and other *Pseudomonas* species from matric or water limitation stresses.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All strains, plasmids, and primers used in this study are listed in Table 1. Cells were cultivated, unless otherwise stated, on tryptone yeast extract (TYE) medium solidified with 10 g of Phytigel (Sigma) liter⁻¹, and the water potential was lowered with various concentrations of polyethylene glycol at a molecular weight (MW) of 8,000 (PEG) to simulate a matric stress or with NaCl or polyethylene glycol at an MW of 200 (PEG 200) to simulate an osmotic stress. To reduce medium water potentials by 0.25, 0.5, 1.0, 1.5, and 2.5 MPa, we added 3.2 or 100 g, 6.4 or 150 g, 12.8 or 262 g, 19.2 or 330 g,

and 32 or 450 g of either NaCl or PEG, respectively, per liter of medium as described previously (15, 45, 46). The defined medium 1/2-strength 21C was prepared as described previously (15).

EPS isolation and quantification. Nylon membranes were placed on TYE plates prior to spreading of a 100- μ l aliquot of 24-h-old surface-grown cultures resuspended in 1 mM phosphate buffer. To maintain the desired relative humidity (RH), plates were incubated in covered plastic containers for 24 h at 27°C. The nylon membrane was removed from the plates, excess agar was scraped off the membrane, and the membrane was then placed in 20 ml of 0.85% saline, vortexed, and centrifuged at $16,300 \times g$ for 30 min at 4°C to separate the EPS from the cells. The EPS-containing supernatant was filtered (pore size, 0.45 μ m) and treated with proteinase K as described previously (35). EPS was precipitated by adding 3 volumes of ethanol (−20°C), incubating overnight at −20°C, and then centrifuging at $16,300 \times g$ for 30 min at 4°C. A second ethanol precipitation was performed on the ethanolic supernatant to ensure adequate EPS recovery (7). EPS was dried at room temperature and resuspended in deionized water. Cell protein content was determined by the method of Bradford (6) with bovine serum albumin as the standard (Bio-Rad). Total-carbohydrate content was determined by the phenol-sulfuric acid method (10) with glucose (Glc) as the standard, and uronic acid content was determined by the *m*-phenylphenol method (5) with D-glucuronic acid (GlcUA) or alginic acid as the standard.

Carbohydrate composition analysis. Carbohydrate composition was analyzed by the Complex Carbohydrate Research Center at the University of Georgia on trimethylsilylated sugars (52). In brief, methyl glycosides were prepared by methanolysis in 1 M HCl in methanol at 80°C (18 h), followed by re-N-acetylation with pyridine and acetic anhydride in methanol for the detection of amino sugars. Samples were then per-O-trimethylsilylated with Tri-Sil (Pierce) at 80°C (0.5 h), separated by gas chromatography using an All Tech EC-1 fused silica capillary column (length, 30 m; inner diameter, 0.25 mm), and analyzed by mass spectrometry with inositol as an internal standard. The amount of each sugar was expressed as the mole percentage of total carbohydrate.

Construction of the *P. putida algD* mutant. We amplified a 622-bp internal region corresponding to nucleotide positions 17 to 644 of the 1.3-kb *algD* gene of *P. putida* KT2440 (30) by colony PCR using primers *algDF* and *algDR* (Table 1). The PCR product was inserted into the XcmI site of pKnockout-G (47), creating pKAD. The identity of the insert was confirmed by sequence analysis. pKAD was first transferred from *Escherichia coli* S17-1 to *P. putida* by conjugation and then integrated into the *algD* gene by a single recombination event resulting in two truncated *algD* genes, as determined by PCR using primers for the *algD* gene and pKnockout-G, pKOF, and pKOR (Table 1).

Biofilm microscopy. Cells were tagged with green fluorescent protein (GFP) by transferring a stable broad-host-range plasmid, pPROBE-KT, containing the *gfp* gene fused to the constitutive neomycin phosphotransferase (*P_{nutI}*) promoter (8). The inoculum was prepared by resuspending cells from a 24-h-old plate culture in TYE prior to inoculation of the solid medium overlying a coverslip with a 1- μ l aliquot containing 10 to 20 cells as described previously (8). The coverslip was placed medium side down on the chamber, taped to the chamber surface, inverted, and incubated at 27°C. Calcofluor white (Sigma), which binds to β -linkages of polysaccharides, was added to the medium at 200 μ g ml^{−1} for visualization of EPS.

Confocal laser scanning microscopy (CLSM) images were obtained using a Leica TCS-NT system equipped with UV and argon lasers for visualization of calcofluor white (emission wavelength, 425 nm; short-cut filter) and GFP (emission wavelength, 510 nm; fluorescein isothiocyanate filter), respectively. Multicolor images were collected simultaneously using a multitrack mode (blue, calcofluor; green, GFP). When calcofluor was included in the medium, we adjusted the sensitivity of the photomultiplier tube of the CLSM until the blue autofluorescence of the medium was no longer detectable.

Horizontal (x-y) images were taken at 0.5- μ m intervals. Vertical cross-section (x-z) images were generated with Image J software (<http://rsb.info.nih.gov/nih-image>). Biofilms were also viewed with a Nikon EFD-3 epifluorescence microscope with a fluorescein isothiocyanate filter set, and images were taken with a SPOT charge-coupled device camera (Diagnostic Instruments). Multiple images were overlaid and cropped using Adobe Photoshop.

Construction of a *PalgD-gfp* transcriptional fusion. The upstream promoter region of the *P. putida algD* gene was PCR amplified from mt-2 genomic DNA with primers *AlgDprm-F* and *AlgDprm-R* (Table 1) to generate a 781-bp promoter region that included the first 10 bases of the *algD* gene (PP1288). PCR conditions were as follows: 25 cycles of 94°C, 54°C, and 72°C for 30 s each, with a final extension time of 2 min at 72°C. The PCR product was first cloned into pTOPO PCR 2.1 (Invitrogen), to generate pTOPO-*PalgD*, and then transformed into *E. coli* TOP10 cells. The insert was sequenced to verify its orientation and identity. pTOPO-*PalgD* was digested with XbaI and SacI, and the resulting

870-bp fragment was isolated prior to cloning into pPROBE-NT (28) containing a promoterless *gfp* gene in order to generate p*PalgD-gfp*. p*PalgD-gfp* was then electroporated into *P. putida* mt-2.

Effects of solute and matrix stresses on *PalgD-gfp* expression. Plate cultures of mt-2(p*PalgD-gfp*), mt-2(p*P_{nutI}-gfp*), and mt-2(pPROBE-NT) were grown overnight on unamended (water-replete) TYE at 28°C and then resuspended in 1/2-strength 21C; subsequently, aliquots were dispensed into 1/2-strength 21C with or without NaCl, ethanol, PEG 200, or PEG amendments to lower the water potential by 1.5 MPa. We used 3.75% ethanol amendments to lower the water potential of the medium by 1.5 MPa (16). Optical density at 600 nm and fluorescence were measured at the beginning of incubation and after the desired incubation period. Cultures were incubated on an orbital shaker at 200 rpm and 28°C. Fluorescence intensity was measured using a Fluoromax-2 spectrofluorometer (Jobin-Yvon-Spex Instruments). Emission and excitation wavelengths, band-pass width, and integration times were 488 nm, 510 nm, 5 nm, and 0.5 s, respectively. A relative fluorescence unit (RFU) is defined as the culture fluorescence relative to the culture optical density at the sampling period. The induction ratio is defined as the RFU for 1.5-MPa water potential reduction treatments divided by the RFU for the water-replete, unamended control.

Matrix stress shock experiment. A 100- μ l aliquot of a 24-h-old surface-grown culture (optical density at 660 nm, 0.001) was inoculated onto a quadrant of an 80-mm-diameter nylon membrane (MSI, Westboro, MA) and then overlaid onto 1/2-strength 21C solid medium in which the water potential was lowered by 1.2 MPa with PEG. Membranes containing biofilms were incubated for 36 h at 24°C or 27°C before being transferred to a solid medium in which the water potential was lowered by 2.5 MPa with PEG or NaCl to create a matrix or osmotic shock. As a control, membranes were also transferred to a −1.2-MPa- ψ PEG-treated medium in order to ascertain the effect of a fresh supply of nutrients on physiological responses. After membrane transfer, biofilms were incubated at 24°C or 27°C until cells were harvested either by resuspending cells from the filters in 1/2-strength 21C broth for ice nucleation activity (INA) assays or by scraping the cells from the filters for fatty acid methyl ester (FAME) analysis. The water potential of the solid medium was verified with a WP4 Dewpoint Potentiometer (Decagon Devices, Pullman, WA) following cell harvest.

INA assay. The water potential-responsive reporter p*P_{ProIce}* contains the *E. coli proU* promoter fused to the ice nucleation gene *inaZ*, as described elsewhere (50). p*P_{NptIce}* served as a constitutive control (50). Ice nuclei were measured at −7.5°C by a droplet freezing assay (26), and the INA was expressed as the number of ice nuclei per cell. Culturable counts were determined with a model D spiral plater (Spiral Systems Instruments) by plating onto Luria-Bertani medium amended with 15 g of agar liter^{−1}.

FAME analysis. Fatty acids were extracted from cells harvested from membranes by mixing the cells with a 15% NaOH solution made in methanol and deionized water (1:1) and then subjecting them to methylation, extraction, and flame ionization detection-gas chromatography using the Sherlock-Microbial Identification System (MIDI Inc.) according to the manufacturer's recommended protocols. Peaks were compared to known standards and 16:1 ω 7t and ω 7c FAME standards (Sigma) as described previously (15). PEG was previously shown not to produce FAME peaks that could be mistaken for fatty acids (15).

Filter disk desiccation assay. Five-microliter aliquots of overnight TYE broth cultures were spotted onto each quartered section of a sterile membrane filter as outlined previously (46). For each time point, two filters were prepared for each strain and transferred to a desiccator containing water or a saturated NaCl solution to generate 100% and 75% RH conditions (48), respectively. Desiccators were kept at room temperature. CFU were determined at various times by resuspending cells from the filter disk in a phosphate buffer and then sonication for 2 min prior to plating dilutions with a model D spiral plater. Desiccation tolerance (percent survival) was calculated as the proportion of culturable bacteria surviving the 75% RH treatment relative to those recovered from the 100% RH condition.

Statistical analyses. Statistical analyses were performed using JMP (version 5; SAS Institute). For comparisons of INA activity, biofilm properties, EPS production, and desiccation survival, an analysis of variance (ANOVA) was performed to determine the significance of the differences as described previously (46). For comparing desiccation survival, we performed separate ANOVA for the CFU from the 100 and 75% RH treatments and for the proportion of cells that survived the desiccation treatment. For comparing matrix stress survival by *P. aeruginosa* and *P. syringae*, a separate ANOVA was performed for each species based on an arcsine square root transformation of the proportion of CFU on the −1.5-MPa-water potential medium relative to that on the water-replete control. Comparisons between treatments were made by Fisher's least-significant-difference (LSD) test, and a *P* value of <0.05 was used as the criterion for statistical significance.

FIG. 1. Effects of NaCl (open symbols) and PEG (solid symbols) treatments on total EPS (squares) and on high-MW (triangles) and uronic acid (circles) components of EPS obtained from *P. putida* mt-2 biofilms. (A) Total and high-MW carbohydrates expressed as Glc equivalents; (B) high-MW uronic acids expressed as GlcUA equivalents. The first ethanol precipitate is referred to as the high-MW EPS, and the sum of the first and second ethanol precipitations is referred to as the total EPS. Values are means $\pm$ standard errors of the means for three to five replications.

RESULTS

Water limitations stimulate EPS production. To simulate a matrix or solute stress, the water potential of the culture medium was lowered with the nonpermeating PEG or permeating NaCl amendment, respectively, as described in Materials and Methods. To evaluate the effects of matrix and solute stress severity on EPS production by *P. putida*, we measured the total-carbohydrate and uronic acid contents of ethanol-precipitated matrix material. With increasing matrix stress severity, there were corresponding increases in total-carbohydrate (Fig. 1A) and uronic acid EPS (Fig. 1B) levels. Most of the increases in total-carbohydrate levels at water potentials lower than -0.5 MPa with PEG treatments were obtained in the second ethanol precipitation, suggestive of the presence of a lower-MW EPS component; there was no corresponding increase in low-MW uronic acid levels (data not shown).

TABLE 3. Uronic acid EPS production by unsaturated *P. putida* mt-2, *P. aeruginosa* PAO1, and *P. syringae* pv. *syringae* B728a biofilms

Strain	Uronic acid EPS production (μg of GlcUA equivalents/mg of protein) ^a at the following ψ (treatment):		
	0 MPa (control)	-1.5 MPa (NaCl)	-1.5 MPa (PEG)
<i>P. putida</i> mt-2	4.6 $\pm$ 0.8 a	1.9 $\pm$ 0.1 a	30.2 $\pm$ 5.9 b
mt-2 <i>algD</i>	4.1 $\pm$ 0.6 a	2.4 $\pm$ 0.3 a	3.7 $\pm$ 0.2 a
<i>P. aeruginosa</i> PAO1	6.5 $\pm$ 1.7 a	3.6 $\pm$ 1.0 a	16.8 $\pm$ 2.3 b
PAO1 <i>algD</i>	3.2 $\pm$ 0.5 a	1.5 $\pm$ 0.6 a	1.9 $\pm$ 0.3 a
<i>P. syringae</i> pv. <i>syringae</i> B728a	2.4 $\pm$ 0.7 a	3.0 $\pm$ 0.2 a	31.2 $\pm$ 1.7 b
B728a <i>algD</i>	2.6 $\pm$ 0.4 a	1.6 $\pm$ 0.1 a	2.9 $\pm$ 0.7 a

^a High-MW EPS obtained from a single ethanol precipitation in two separate experiments. Values are means $\pm$ standard errors of the means for three to six replicates. The wild type strains mt-2, PAO1, and B728a produced significantly ($P < 0.05$) more uronic acids than their corresponding *algD* mutants. A separate ANOVA was performed for each species. Values followed by the same letter are not statistically different ($P < 0.05$) based on Fisher's LSD test.

Alginate is an EPS component under matrix stress conditions. Analysis of the composition of the EPS revealed that mannuronic acid was a matrix component only when wild-type *P. putida* mt-2 was grown on a PEG-amended medium (Table 2); *Pseudomonas* species appear to make alginates composed exclusively of mannuronic acid (4, 49). In contrast, no mannuronic acid was present in the EPS of mt-2 *algD* (Table 2), which had the alginate biosynthesis operon disrupted by site-specific mutagenesis, nor did the amount of uronic acid-containing material increase on PEG-treated medium (Table 3). Low levels of uronic acids in the EPS isolated from mt-2 *algD* with PEG treatment are likely due to the presence of a glucuronic acid-containing constituent (Table 2). In the absence of a matrix stress, the compositions of the EPSs of mt-2 and mt-2 *algD* were nearly identical (Table 2). The EPS of mt-2 contained more Glc and less rhamnose in cultures under matrix stress than in unstressed cultures (Table 2), suggesting that other, unknown EPSs are made in response to matrix stress. The presence of rhamnose could be due to the presence of lipopolysaccharide in our samples, although we did not detect

TABLE 2. Glycosyl composition of *P. putida* biofilm EPS^a

Glycosyl residue	Amt of sugar (mol% of total carbohydrate) at the following ψ (treatment):					
	0 MPa (control)		-1.5 MPa (NaCl)		-1.5 MPa (PEG)	
	mt-2	mt-2 <i>algD</i>	mt-2 ^b	mt-2 <i>algD</i>	mt-2	mt-2 <i>algD</i>
Glucose	39.6	40.5	78.2	ND	62.4	54.1
Rhamnose	42.4	38.5	7.3	ND	5.8	24.9
Mannose	9.4	9.3	14.5	ND	4.4	10.7
Glucuronic acid	8.6	9.4	0.0	ND	0.0	6.8
Mannuronic acid	0.0	0.0	0.0	ND	27.4	0.0
Xylose	0.0	2.3	0.0	ND	0.0	3.5

^a Values are representative of two separate analyses, each consisting of three replications that were pooled for analysis. ND, not determined.

^b Values for mt-2 at a ψ of -1.5 MPa (NaCl) represent analysis of pooled first and second ethanol precipitations.

2-keto-3-deoxyoctonate in our glycosyl composition analyses (Table 2) or in separate biochemical assays (data not shown). Comparison of the uronic acid contents of mt-2 and mt-2 *algD* cultivated in the presence or absence of $-1.5\text{-MPa-}\psi$ solute stress (NaCl or PEG 200) or matric stress (PEG) treatments, in the presence of toluene (aqueous-phase concentration, 100 $\mu\text{g/ml}$), or in cold temperatures (15°C) showed that only mt-2 exposed to the $-1.5\text{-MPa-}\psi$ PEG treatment resulted in alginate production (data not shown).

Similarly, for *P. aeruginosa* PAO1 and *P. syringae* B728a, but not for their corresponding *algD* mutants, there were 2- to 4-fold more total EPS (data not shown) and 5- to 10-fold more uronic acids with the matric stress treatment than with the water-replete or solute stress treatments (Table 3). Increased EPS production with the matric stress treatments was not a consequence of selection for mucoidy variants, because cells cultivated at a water potential of -1.5 MPa with PEG treatment exhibited nonmucoidy phenotypes when subsequently cultivated under water-replete conditions. Although not exhaustive, our results strongly suggest that one common adaptive response to matric stress by unsaturated *Pseudomonas* biofilms is alginate production.

Alginate gene expression. Previous studies have suggested that *algD* gene expression occurs in a strain- and species-specific manner in response to a variety of environmental stimuli (3, 9, 11, 23, 27, 38, 42), although alginate production is not always observed. To further explore the role of alginate, we examined the effects of reductions in water potential on *algD* operon expression in *P. putida*. We constructed mt-2(p*PalgD-gfp*) and mt-2(p*PnptII-gfp*), which harbor ectopic plasmid fusions of the promoters for *algD* and *nptII*, respectively. We examined the role of 1.5-MPa water potential reductions imposed by solute (NaCl, PEG 200, or ethanol) or matric (PEG) stress. Unsaturated biofilms were cultivated under water-replete conditions before cells were exposed to liquid cultures containing the various amendments. As shown in Fig. 2, there was no significant effect of reductions in water potential imposed by any of the permeating solutes on *algD* expression, whereas there was a dramatic increase in *algD* expression within 6 h after exposure to the PEG treatment. There was a slight increase in the expression of the constitutive control mt-2(p*PnptII-gfp*) following exposure to the PEG treatment, indicating that PEG slightly influences cell fluorescence intensity (Fig. 2), although its effect is small compared to the maximum level of *algD* expression. Expression by the promoterless *gfp* negative control was unaffected by treatments imposing reductions in water potential (data not shown).

Alginate production alters biofilm ultrastructure. To further understand the role of alginate, we investigated whether the ability to produce alginate influenced biofilm development. CLSM and epifluorescence microscopy of GFP-tagged mt-2 and mt-2 *algD* revealed that the dynamics of microcolony formation under water-replete and solute stress conditions were indistinguishable from each other, leading to biofilms with similar features (data not shown). In contrast, with the PEG treatments, within 12 h mt-2 *algD* biofilms exhibited surface colonization patterns different from those of mt-2 (Fig. 3A and E), leading to biofilms that covered more surface area (Fig. 3B and F) with a higher cell density (Fig. 3C and G) and were shorter, with a thinner EPS layer at the air interface (Fig. 3D

FIG. 2. Effects of reductions in water potential (-1.5 MPa) imposed by permeating (NaCl, PEG 200, or ethanol) solutes or by PEG on *algD* expression. The induction ratio is defined as the fluorescence observed in the treated cultures relative to that in water-replete controls. Solid symbols, p*PalgD-gfp*NT; open symbols, p*PnptII-gfp*_{AAV}NT; circles, PEG; squares, PEG 200; diamonds, ethanol; triangles, NaCl. Error bars (standard errors of the means) that are not visible are masked by the symbols.

and H; Table 4). These findings indicate that alginate influences biofilm architecture under water-limiting conditions.

Alginate decreases the extent of cell drying. We used two approaches to assess whether alginate creates a more hydrated microenvironment that protects cells by slowing the rate or extent of cellular drying. Biofilms were generated on membranes overlying a solid medium in which the water potential was lowered by 1.2 MPa with PEG amendments, since this condition favored alginate production (Fig. 1). We transferred the nylon membrane harboring intact biofilms of approximately 10,000 cells per microcolony to solid media of equivalent water potentials or to plates with water potentials lowered by 2.5 MPa with PEG or NaCl to create a matric or solute shock, respectively. We then assayed the intracellular water potential of biofilm cells, by using a *proU-inaZ* transcriptional fusion (50), or the extent of membrane dehydration, by monitoring the *cis*-to-*trans* isomerase activity of membrane fatty acids, indicative of dehydration stress (15, 24).

Plasmid pPProIce, which harbors a transcriptional fusion between the *E. coli proU* promoter, responsive to both the solute and matric components of water potential, and the ice-nucleating protein *inaZ* has been used as a tool for quantifying the water potential sensed by individual bacterial cells (2, 50). We transferred this plasmid to *P. putida*, and the INAs of mt-2(pPProIce) and mt-2 *algD*(pPProIce) were directly related to the water potential to which the cells were exposed during growth (Fig. 4), indicating that we can use pPProIce as a tool to monitor intracellular water potential. Within 2.5 h post-transfer, mt-2 *algD*(pPProIce) cells exhibited significantly higher ($P < 0.05$) INA than mt-2(pPProIce) cells when exposed to a matric, but not a solute, stress shock (Fig. 5A). There was no significant difference ($P > 0.05$) between the INAs of mt-2(pPProIce) and mt-2 *algD*(pPProIce) cells following transfer to a fresh medium of the same water potential (Fig. 5A) throughout the duration of the experiment. Additionally, the constitutive control strains mt-2(pPNptIce) and mt-2 *algD*(pPNptIce) did not exhibit significant changes in INA over time following the matric or solute stress shock treatments (Fig. 5B), indicating that changes in INA following

FIG. 3. Epifluorescence microscopy (A, B, E, and F) and CLSM (C, D, G, and H) of the temporal dynamics of biofilm development by *gfp*-tagged *P. putida* mt-2 (A to D) and mt-2 *algD* (E to H) with $-1.5\text{-MPa-}\psi$ PEG treatments. Sagittal images (D and H) were created from a collection of 50 to 55 images at a $0.5\text{-}\mu\text{m}$ interval. Blue, calcofluor-stained material. hpi, hours postinoculation.

these shocks are not due to physiological changes that alter INA. By using the linear relations between INA and water potential on a solid medium (Fig. 4), we estimated the intracellular water potentials of cells following the matric and solute stress shock treatments. By 4 h posttransfer, the intracellular water potential of mt-2(pPProIce) cells (-2.28 ± 0.03 MPa) was significantly ($P < 0.05$) higher than that of mt-2 *algD*-(pPProIce) cells (-2.50 ± 0.03 MPa) with the $-2.5\text{-MPa-}\psi$ PEG treatments, while there was no significant difference ($P > 0.05$) between the intracellular water potentials of mt-2 and the mt-2 *algD* mutant with the $-1.2\text{-MPa-}\psi$ PEG or $-2.5\text{-MPa-}\psi$ NaCl treatment (Fig. 5C). These results suggest that the pres-

ence of alginate keeps the cells more hydrated following a desiccation event.

cis-to-*trans* isomerization was previously shown to increase in response to matric but not osmotic stress, presumably to counter desiccation-mediated membrane stress (15, 24), and to occur in response to an abrupt disturbance, precluding de novo synthesis of fatty acids (17). Within 30 min of the matric stress shock, there was a statistically significant increase ($P < 0.05$) in the 16:1 ω 7 *trans/cis* ratio by mt-2 *algD*, but not by mt-2 (Fig. 6): a 20% increase in the *trans* isomer. There was no change in the 16:1 ω 7 *trans/cis* ratio in mt-2 or mt-2 *algD* following a thermodynamically equivalent osmotic shock (data not shown). In the isotonic control treatments (-1.2-MPa to -1.2-MPa water potential treatments), the 16:1 *trans/cis* ratios of both strains decreased dramatically (Fig. 6), suggesting that fresh nutrients allow for the growth of new cells with lower *trans* fatty acid contents or the conversion of preexisting 16:1 *trans* fatty acids to the *cis* isomer (15). During steady-state growth in the absence or presence of $-1.5\text{-MPa-}\psi$ PEG or NaCl treatments,

TABLE 4. Biofilm properties of <i>P. putida</i> mt-2 and mt-2 <i>algD</i> ^a				
Water potential (treatment) and strain	Microcolony surface area (μm^2) ^b	Biofilm cell ht (μm) ^c	EPS thickness at air interface (μm) ^d	Density (cells/100 μm^2) ^e
0 MPa (control)				
mt-2	61,427 $\pm$ 6,300 a	9.3 $\pm$ 0.6 a	2.5 $\pm$ 0.3 a	117 $\pm$ 1 a
mt-2 <i>algD</i>	65,765 $\pm$ 1,413 a	9.2 $\pm$ 0.3 a	2.6 $\pm$ 0.1 a	116 $\pm$ 2 a
-1.5 MPa (PEG)				
mt-2	4,162 $\pm$ 700 c	17.3 $\pm$ 0.8 c	5.8 $\pm$ 0.5 c	70 $\pm$ 2 c
mt-2 <i>algD</i>	7,189 $\pm$ 370 b	12.2 $\pm$ 0.4 b	3.4 $\pm$ 0.2 b	81 $\pm$ 2 b

^a Values are means $\pm$ standard errors of the means. A separate ANOVA was performed for each biofilm property. Values followed by the same letter within a column are not statistically different ($P < 0.05$) based on Fisher's LSD test.

^b At the substratum surfaces of 1-day-old biofilms. Values were derived from the average microcolony surface area per field of view ($n = 4$) containing 5 to 33 microcolonies.

^c Height from the substratum to the top of biofilm, derived from 9 random height measurements of 10 to 13 x - z projections of a stacked series of x - y plane CLSM images of 1-day-old biofilms.

^d Thickness of the calcofluor-stained layer between the top of the biofilm and the air interface.

^e In 5-day-old biofilms; derived from 13 to 21 x - y plane CLSM images per replication.

FIG. 4. Correlation between the INA of cells and water potential. Cells were cultivated on solid medium amended with various concentrations of PEG to achieve the desired water potential. Solid symbols, mt-2(pPProIce); open symbols, mt-2 *algD*(pPProIce).

FIG. 5. Temporal dynamics of INA and estimated intracellular water potentials following matric or osmotic shock treatments. (A) mt-2(pPProlce) and mt-2 *algD*(pPProlce); (B) constitutive controls mt-2(pPNptlce) and mt-2 *algD*(pPNptlce); (C) estimated intracellular water potential. Cells were cultivated on $-1.2\text{-MPa-}\psi$ PEG-amended medium for 36 h prior to transfer to $-1.2\text{-MPa-}\psi$ PEG (triangles), $-2.5\text{-MPa-}\psi$ PEG (circles), or $-2.5\text{-MPa-}\psi$ NaCl (squares)-amended medium. Solid symbols, mt-2; open symbols, mt-2 *algD*. Values are means $\pm$ standard errors of the means for two or three experiments, each comprising three replications.

there were no statistically significant differences ($P > 0.05$) in the 16:1 *trans/cis* ratios of mt-2 and mt-2 *algD*, indicating that the fatty acid composition of mt-2 *algD* was not inherently different from that of mt-2 (data not shown). Taken together,

FIG. 6. 16:1 *trans/cis* fatty acid ratios of mt-2 (solid symbols) and mt-2 *algD* (open symbols) following a matric stress shock experiment. Levels of fatty acids are expressed relative to their abundance before the matric stress shock. Triangles, $-1.2\text{-MPa-}\psi$ PEG control treatment; circles, $-2.5\text{-MPa-}\psi$ PEG dehydration shock treatment. Values are means $\pm$ standard errors of the means (three replications per experiment) from one of two separate experiments with similar results.

FIG. 7. Desiccation tolerances of mt-2 (solid symbols) and mt-2 *algD* (open symbols). Circles (left y axis), CFU at 100% RH. Triangles (right y axis), percent survival at 75% RH relative to survival at 100% RH (taken as 100% survival). Values are means $\pm$ standard errors of the means from three replications.

the data suggest that alginate reduces the extent of *cis*-to-*trans* isomerase activity necessary to counter the effects of matric stress on membrane integrity.

Alginate contributes to desiccation tolerance. To determine whether alginate contributes to desiccation tolerance, we exposed planktonic cells to a 100% or 75% RH environment and monitored the CFU over time. Previously we had shown that with the 75% RH treatment, significant decreases in water potential occurred within 6 to 24 h following initiation of the desiccation treatment, providing ample time for biofilms to form (45). Following a 6-h acclimation period in which there was no substantial change in the CFU of mt-2 and mt-2 *algD*, there was a >10 -fold increase in population sizes by 24 h (Fig. 7). However, after 24 h, population sizes gradually decreased in desiccated samples, and by 120 h, mt-2 survival in desiccated samples was 13% of that in undesiccated samples whereas less than 1% of the mt-2 *algD* population survived (Fig. 7). Similarly, *algD* mutants of both *P. aeruginosa* PAO1 and *P. syringae* pv. *syringae* B728a were statistically significantly ($P < 0.05$) less tolerant of matric stress, but not of solute stress, than the corresponding wild-type strains (Table 5). Taken together, these results suggest that alginate contributes to the tolerance of desiccation stress by various *Pseudomonas* species.

DISCUSSION

In this study, we provide two lines of evidence that one function of alginate is to create a more hydrated microenvironment that buffers biofilm cells from drying but gives no protection from osmotic stresses. First, *P. putida* cells encap-

TABLE 5. Tolerance of solute and matric stresses by *P. aeruginosa* PAO1 and *P. syringae* pv. *syringae* B728a

Water potential (treatment)	% Survival relative to control ^a			
	<i>P. aeruginosa</i>		<i>P. syringae</i> pv. <i>syringae</i>	
	PAO1	PAO1 <i>algD</i>	B728a	B728a <i>algD</i>
-1.5 MPa (NaCl)	84.9 $\pm$ 6.1 a	87.4 $\pm$ 4.9 a	86.6 $\pm$ 5.3 a	75.7 $\pm$ 5.3 a,b
-1.5 MPa (PEG)	68.0 $\pm$ 8.9 a	29.7 $\pm$ 9.0 b	48.5 $\pm$ 9.6 b	13.5 $\pm$ 7.2 c

^a Values are means $\pm$ standard errors of the means for six to nine replications. Survival is based on the CFU on the NaCl- or PEG-amended medium relative to the CFU for the water-replete control. Eight 5- μ l aliquots of dilutions of cells cultivated under water-replete conditions were placed on each medium to assess survival. For each species, values followed by the same letter are not statistically different ($P = 0.05$), based on Fisher's LSD test.

sulated with alginate perceive less water stress than those without alginate (mt-2 *algD*) following a matric stress shock as indicated by the intracellular water potential biosensor, pPProIce (Fig. 5). Second, alginate decreased the extent of membrane dehydration, as evidenced by the increase in the level of *trans* unsaturated fatty acids by mt-2 *algD* compared to the wild-type level following a matric stress shock (Fig. 6). Alginate production by *P. putida* under water-limiting conditions is regulated at the transcriptional level, as indicated by the dramatic increase in *algD* expression observed with matric stress, but not solute stress, treatments (Fig. 2), which is consistent with our biochemical analysis of biofilm EPS (Fig. 1; Table 3). Moreover, we also show that alginate is integral to biofilm architecture under matric stress but not under solute stress or water-replete conditions. This is in contrast to the suggestion that alginate is not a significant component of *P. aeruginosa* biofilms in saturated flowthrough systems (49) and highlights how normal biofilm developmental processes are modulated by environmental stresses. Biofilms formed by *P. putida* mt-2 under water-limiting conditions were architecturally similar (taller and more compact) to those formed by the alginate-overproducing *P. aeruginosa* strain PDO300 in a flowthrough biofilm system (18), illustrating the important role alginate plays in modifying biofilm architecture. Ultimately, desiccation tolerance may be due to alginate's hygroscopic properties or to its influence in developing a biofilm architecture that may reduce evaporative loss, or both.

The relative importance of alginate for tolerance needs to be viewed with the understanding that it is not the primary EPS component under matric stress conditions. Composition analysis shows other glycosyl residue changes in addition to the production of alginate, most notably an increase in the level of a Glc-containing constituent(s) that coincides with decreases in the levels of others (rhamnose and GlcUA). The extent to which the presence or absence of other EPS constituents produced under water-limiting conditions contributes to biofilm development and desiccation tolerance is currently being explored. Given that alginate constituted only 10 to 40% of the high-MW EPS fractions of the various *Pseudomonas* species we examined (Fig. 1 and Tables 2 and 3; also data not shown), our findings highlight how a relatively small amount of alginate can protect biofilm cells from desiccation stress or modulate biofilm architecture. Since EPS is a shared resource (51), the production of a small amount of alginate by a subset of biofilm residents could greatly contribute to the desiccation tolerance of residents or to biofilm architecture in single-species or multispecies biofilms.

Other studies have implicated EPS matrix components (21, 36, 39, 53) in desiccation tolerance, although direct evidence demonstrating that the presence of specific constituents increases cellular hydration during desiccation events has been lacking. Most notably, Ophir and Gutnick found that colanic acid protects planktonic enteric bacteria from desiccation imposed by a vacuum (31), which results in nearly complete removal of water from a system, but this study did not address how the biofilm lifestyle may modulate the importance of colanic acid for tolerance. We chose to investigate the interrelationships between biofilm growth, alginate production itself (rather than a global regulator for alginate production), and stress tolerance under levels of water unsaturation that com-

monly occur in many terrestrial ecosystems and to contrast these responses to those under water-replete or high-osmolarity conditions. As a result, we were able to reveal the intricate interrelationship between adaptation to water limitation stress and biofilm developmental processes in creating a biofilm microenvironment that likely contributes to desiccation stress survival.

Alginate is hygroscopic; it can hold several times its weight in water (37, 44) and potentially loses water slowly, thereby keeping cells hydrated long enough to allow them to make the metabolic adjustments necessary to enhance desiccation stress survival. Our findings indicate that alginate does retain water in the cell microenvironment (Fig. 5 and 6) either directly, due to its hygroscopic properties, or through its ability to influence the development of a biofilm architecture that reduces evaporative water loss, or both. However, the smaller microcolony surface area of wild-type mt-2 relative to that of mt-2 *algD* (Table 4) under water-limiting conditions likely does not lead to reduced evaporative water loss that increases desiccation tolerance (Fig. 7). Given that 25 to 50% of the inoculum forms microcolonies in the desiccation assay and we assume that all cells are deposited as individuals, biofilm microcolonies comprise, on average, 23 to 500 cells, a density that does not likely possess the highly structured 3-dimensional architecture of fully developed microcolonies. This suggests that even small aggregates may produce sufficient amounts of alginate to protect cells from desiccation stress, although tolerance is likely improved with increased aggregate size and biofilm ultrastructural complexity. Similarly, Monier and Lindow (29) suggested that aggregate size is an important factor influencing *P. syringae* survival on leaves, with aggregates containing 10^3 cells or more accounting for the majority of cells on leaves surviving a desiccation stress.

Collectively, our findings reveal that *P. putida*, and likely other pseudomonads, integrates cues on water abundance into regulatory networks controlling stress adaptation and biofilm developmental processes, interacting in a fashion that promotes survival in water-limited environments. The prevalence of alginate biosynthesis capabilities among pseudomonads (13) suggests that alginate is an important fitness trait under certain environmental conditions (e.g., drought) or in particular habitats (e.g., rhizosphere, aerial leaf surfaces, or the CF lung) (9, 34) that can, at times, be water limited. Interestingly, evidence of alginate gene expression by *P. syringae* pv. tomato DC3000 in planta (22) preceding the hypersensitive response (HR) coincides with inhibitory levels of water stress during the HR (50). It is conceivable that in addition to or coincident with reactive oxygen species-mediated induction of alginate synthesis in planta, matric (water limitation) stress occurs during the initial stages of the HR. Additionally, our findings suggest that one potential factor contributing to selection for mucoid *P. aeruginosa* variants in the CF lung environment is matric stress-mediated selection for alginate overproducers that could increase not only their own fitness but also that of neighboring bacterial cells. Alginate production could clearly provide a competitive advantage in water-limited environments, leading to increased ecological success.

ACKNOWLEDGMENTS

We thank Gwyn Beattie for providing pPProIce and pPNptIce, Matt Parsek for providing PAO1 *algD*, and Erin Parker and Nicole O'Tool for technical assistance. We thank G. Beattie, William L. Franck, and M. Parsek for reading a draft of the manuscript.

This material is based on work supported by the National Science Foundation under grant 0446292, by the Mary and Raymond Baker Family Trust, and by the Center for Plant and Microbial Complex Carbohydrates, funded by the Department of Energy.

REFERENCES

- Auerbach, I. D., C. Sorensen, H. G. Hansma, and P. A. Holden. 2000. Physical morphology and surface properties of unsaturated *Pseudomonas putida* biofilms. *J. Bacteriol.* **182**:3809–3815.
- Axtell, C. A., and G. A. Beattie. 2002. Construction and characterization of a *proU-gfp* transcriptional fusion that measures water availability in a microbial habitat. *Appl. Environ. Microbiol.* **68**:4604–4612.
- Berry, A., J. D. DeVault, and A. M. Chakrabarty. 1989. High osmolarity is a signal for enhanced *algD* transcription in mucoid and nonmucoid *Pseudomonas aeruginosa* strains. *J. Bacteriol.* **171**:2312–2317.
- Bjerkkan, T. M., C. L. Bender, H. Ertesvag, F. Drablos, M. K. Fakhr, L. A. Preston, G. Skjak-Braek, and S. Valla. 2004. The *Pseudomonas syringae* genome encodes a combined mannuronan C-5-epimerase and O-acetylhydrolase, which strongly enhances the predicted gel-forming properties of alginates. *J. Biol. Chem.* **279**:28920–28929.
- Blumenkrantz, N., and G. Asboe-Hansen. 1973. New method for quantitative determination of uronic acids. *Anal. Biochem.* **54**:484–489.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
- Breedveld, M. W., L. P. T. M. Zevenhuizen, and A. J. B. Zehnder. 1991. Osmotically-regulated trehalose accumulation and cyclic β -(1,2)-glucan excretion by *Rhizobium leguminosarum* biovar trifolii TA-1. *Arch. Microbiol.* **156**:501–506.
- Chang, W.-S., and L. J. Halverson. 2003. Reduced water availability influences the dynamics, development, and ultrastructural properties of *Pseudomonas putida* biofilms. *J. Bacteriol.* **185**:6199–6204.
- DeVault, J. D., K. Kimbara, and A. M. Chakrabarty. 1990. Pulmonary dehydration and infection in cystic fibrosis: evidence that ethanol activates alginate gene expression and induction of mucoidy in *Pseudomonas aeruginosa*. *Mol. Microbiol.* **4**:737–745.
- Dubois, M., K. A. Giles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28**:350–356.
- Edwards, K. J., and N. A. Saunders. 2001. Real-time PCR used to measure stress-induced changes in the expression of the genes of the alginate pathway of *Pseudomonas aeruginosa*. *J. Appl. Microbiol.* **91**:29–37.
- Fett, W. F., J. M. Wells, P. Cescutti, and C. Wijey. 1995. Identification of exopolysaccharides produced by fluorescent pseudomonads associated with commercial mushroom (*Agaricus bisporus*) production. *Appl. Environ. Microbiol.* **61**:513–517.
- Fialho, A. M., N. A. Zielinski, W. F. Fett, A. M. Chakrabarty, and A. Berry. 1990. Distribution of alginate gene sequences in the *Pseudomonas* rRNA homology group I-Azomonas-Azotobacter lineage of superfamily B pro-caryotes. *Appl. Environ. Microbiol.* **56**:436–443.
- Figurski, D. H., and D. R. Helinski. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. *Proc. Natl. Acad. Sci. USA* **76**:1648–1652.
- Halverson, L. J., and M. K. Firestone. 2000. Differential effects of permeating and nonpermeating solutes on the fatty acid composition of *Pseudomonas putida*. *Appl. Environ. Microbiol.* **66**:2414–2421.
- Harris, R. F. 1980. Effect of water potential on microbial growth and activity, p. 23–95. In J. F. Parr, W. R. Gardner, and L. F. Elliott (ed.), *Water potential relations in soil microbiology*. Soil Science Society of America, Madison, WI.
- Härtig, C., N. Löffhagen, and H. Harms. 2005. Formation of *trans* fatty acids is not involved in growth-linked membrane adaptation of *Pseudomonas putida*. *Appl. Environ. Microbiol.* **71**:1915–1922.
- Hentzer, M., G. M. Teitzel, G. J. Balzer, A. Heydorn, S. Molin, M. Givskov, and M. R. Parsek. 2001. Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. *J. Bacteriol.* **183**:5395–5401.
- Jackson, K. D., M. Starke, S. Kremer, M. R. Parsek, and D. J. Wozniak. 2004. Identification of *psl*, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. *J. Bacteriol.* **186**:4466–4475.
- Kachlany, S. C., S. B. Levery, J. S. Kim, B. L. Reuhs, L. W. Lion, and W. C. Ghiorse. 2001. Structure and carbohydrate analysis of the exopolysaccharide capsule of *Pseudomonas putida* G7. *Environ. Microbiol.* **3**:774–784.
- Keith, L. M., and C. L. Bender. 1999. AlgT (σ^{22}) controls alginate production and tolerance to environmental stress in *Pseudomonas syringae*. *J. Bacteriol.* **181**:7176–7184.
- Keith, R. C., L. M. W. Keith, G. Hernandez-Guzman, S. R. Uppalapati, and C. L. Bender. 2003. Alginate gene expression by *Pseudomonas syringae* pv. tomato DC3000 in host and non-host plants. *Microbiology* **149**:1127–1138.
- Kidambi, S. P., G. W. Sundin, D. A. Palmer, A. M. Chakrabarty, and C. L. Bender. 1995. Copper as a signal for alginate synthesis in *Pseudomonas syringae* pv. *syringae*. *Appl. Environ. Microbiol.* **61**:2172–2179.
- Kieft, T., D. Ringelberg, and D. White. 1994. Changes in ester-linked phospholipid fatty acid profiles of subsurface bacteria during starvation and desiccation in a porous medium. *Appl. Environ. Microbiol.* **60**:3292–3299.
- Laue, H., A. Schenk, H. Li, L. Lambertsens, T. R. Neu, S. Molin, and M. S. Ullrich. 2006. Contribution of alginate and levan production to biofilm formation by *Pseudomonas syringae*. *Microbiology* **152**:2909–2918.
- Lindow, S. E., D. C. Army, and C. D. Upper. 1978. *Erwinia herbicola*—bacterial ice nucleus active in increasing frost injury to corn. *Phytopathology* **68**:523–527.
- Ma, J. F., P. V. Phibbs, and D. J. Hassett. 1997. Glucose stimulates alginate production and *algD* transcription in *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* **148**:217–221.
- Miller, W. G., J. H. Leveau, and S. E. Lindow. 2000. Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. *Mol. Plant-Microbe Interact.* **13**:1243–1250.
- Monier, J.-M., and S. E. Lindow. 2004. Frequency, size, and localization of bacterial aggregates on bean leaf surfaces. *Appl. Environ. Microbiol.* **70**:346–355.
- Nelson, K. E., C. Weinle, I. T. Paulsen, R. J. Dodson, H. Hilbert, V. A. Martins dos Santos, D. E. Fouts, S. R. Gill, M. Pop, M. Holmes, L. Brinkac, M. Beanan, R. T. DeBoy, S. Daugherty, J. Kolonay, R. Madupu, W. Nelson, O. White, J. Peterson, H. Khouri, I. Hance, P. Chris Lee, E. Holtzapple, D. Scanlan, K. Tran, A. Moazzes, T. Utterback, M. Rizzo, K. Lee, D. Kosack, D. Moestl, H. Wedler, J. Lauber, D. Stjepandic, J. Hoheisel, M. Straetz, S. Heim, C. Kiewitz, J. A. Eisen, K. N. Timmis, A. Dusterhoft, B. Tummeler, and C. M. Fraser. 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.* **4**:799–808.
- Ophir, T., and D. Gutnick. 1994. A role for exopolysaccharides in the protection of microorganisms from desiccation. *Appl. Environ. Microbiol.* **60**:740–745.
- Papendick, R. L., and G. S. Campbell. 1980. Theory and measurement of water potential, p. 1–22. In J. F. Parr, W. R. Gardner, and L. F. Elliott (ed.), *Water potential relations in soil microbiology*. Soil Science Society of America, Madison, WI.
- Priester, J. H., S. G. Olson, S. M. Webb, M. P. Neu, L. E. Hersman, and P. A. Holden. 2006. Enhanced exopolymer production and chromium stabilization in *Pseudomonas putida* unsaturated biofilms. *Appl. Environ. Microbiol.* **72**:1988–1996.
- Ramos-González, M. I., M. J. Campos, and J. L. Ramos. 2005. Analysis of *Pseudomonas putida* KT2440 gene expression in the maize rhizosphere: in vivo expression technology capture and identification of root-activated promoters. *J. Bacteriol.* **187**:4033–4041.
- Read, R. R., and J. W. Costerton. 1987. Purification and characterization of adhesive exopolysaccharides from *Pseudomonas putida* and *Pseudomonas fluorescens*. *Can. J. Microbiol.* **33**:1080–1090.
- Roberson, E., and M. Firestone. 1992. Relationship between desiccation and exopolysaccharide production in a soil *Pseudomonas* sp. *Appl. Environ. Microbiol.* **58**:1284–1291.
- Robyt, J. F. 1998. *Essentials of carbohydrate chemistry*, p. 157–227. Springer-Verlag, New York, NY.
- Sabra, W., A.-P. Zeng, H. Lunsdorf, and W.-D. Deckwer. 2000. Effect of oxygen on formation and structure of *Azotobacter vinelandii* alginate and its role in protecting nitrogenase. *Appl. Environ. Microbiol.* **66**:4037–4044.
- Schneider-Keel, U., K. B. Lejbolle, E. Baehler, D. Haas, and C. Keel. 2001. The sigma factor AlgU (AlgT) controls exopolysaccharide production and tolerance towards desiccation and osmotic stress in the biocontrol agent *Pseudomonas fluorescens* CHA0. *Appl. Environ. Microbiol.* **67**:5683–5693.
- Simon, R., U. Priefer, and A. Puhler. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Bio/Technology* **1**:784–791.
- Singh, P. K., A. L. Schaefer, M. R. Parsek, T. O. Moninger, M. J. Welsh, and E. P. Greenberg. 2000. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* **407**:762–764.
- Singh, S., B. Koehler, and W. F. Fett. 1992. Effect of osmolarity and dehydration on alginate production by fluorescent pseudomonads. *Curr. Microbiol.* **25**:335–339.
- Steinberger, R. E., A. R. Allen, H. G. Hansa, and P. A. Holden. 2002. Elongation correlates with nutrient deprivation in *Pseudomonas aeruginosa* unsaturated biofilms. *Microb. Ecol.* **43**:416–423.
- Sutherland, I. 2001. Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology* **147**:3–9.
- van de Mortel, M., W.-S. Chang, and L. J. Halverson. 2004. Differential tolerance of *Pseudomonas putida* biofilm and planktonic cells to desiccation. *Biofilms* **1**:361–368.
- van de Mortel, M., and L. J. Halverson. 2004. Cell envelope components

- contributing to biofilm growth and survival of *Pseudomonas putida* in low-water-content habitats. *Mol. Microbiol.* **52**:735–750.
47. Windgassen, M., A. Urban, and K. E. Jaeger. 2000. Rapid gene inactivation in *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* **193**:201–205.
 48. Winston, P. W., and D. H. Bates. 1960. Saturated solutions for the control of humidity in biological research. *Ecology* **41**:232–237.
 49. Wozniak, D. J., T. J. Wyckoff, M. Starkey, R. Keyser, P. Azadi, G. A. O'Toole, and M. R. Parsek. 2003. Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. *Proc. Natl. Acad. Sci. USA* **100**:7907–7912.
 50. Wright, C. A., and G. A. Beattie. 2004. *Pseudomonas syringae* pv. tomato cells encounter inhibitory levels of water stress during the hypersensitive response of *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **101**:3269–3274.
 51. Xavier, J. B., and K. R. Foster. 2007. Cooperation and conflict in microbial biofilms. *Proc. Natl. Acad. Sci. USA* **104**:876–881.
 52. York, W. S., A. G. Darvill, M. McNeil, T. T. Stevenson, and P. Albersheim. 1986. Isolation and characterization of plant-cell walls and cell-wall components. *Methods Enzymol.* **118**:3–40.
 53. Yu, J., A. Penaloza-Vazquez, A. M. Chakrabarty, and C. L. Bender. 1999. Involvement of the exopolysaccharide alginate in the virulence and epiphytic fitness of *Pseudomonas syringae* pv. *syringae*. *Mol. Microbiol.* **33**:712–720.